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Abegg, Kathrin ; Bernasconi, Lara ; Hutter, Melanie ; Whiting, Lynda ; Pietra, Claudio ; Giuliano, Claudio ; Lutz, Thomas A ; Riediger, Thomas

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Ghrelin receptor inverse agonists as a novel therapeutic approach against obesity-related metabolic disease

Running title: Therapeutic ghrelin receptor inverse agonists

Kathrin Abegg [1], Lara Bernasconi [1], Melanie Hutter [1], Lynda Whiting [1], Claudio Pietra [3], Claudio Giuliano [3] , Thomas A. Lutz [1,2], Thomas Riediger [1,2]

[1] Institute of Veterinary Physiology, University of Zurich, Switzerland

[2] Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland

[3] Helsinn SA, Research and Preclinical Development Department, Lugano, Switzerland

Corresponding author:

Thomas Riediger

University of Zurich

Institute of Veterinary Physiology

Winterthurerstrasse 260

8057 Zurich, Switzerland

Phone: +41-44-635-8815

Fax: +41-44-635-8932

e-mail: triedig@vetphys.uzh.ch

Abstract

Aims:

Ghrelin is implicated in the control of energy balance and glucose homeostasis. The ghrelin receptor exhibits ligand independent constitutive activity, which can be pharmacologically exploited to induce inverse ghrelin actions. Since ghrelin receptor inverse agonists (GHSR-IA) might be effective for the treatment of obesity-related metabolic disease, we tested two novel synthetic compounds GHSR-IA1 and GHSR-IA2.

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Materials and methods:

In functional cell assays, electrophysiogical and immunohistochemical experiments, we demonstrated inverse agonist activity for GHSR-IA1 and GHSR-IA2. We used healthy mice, Zucker diabetic fatty (ZDF) rats and diet-induced obese mice (DIO) to explore effects on food intake (FI), body weight (BW), conditioned taste aversion (CTA), oral glucose tolerance (OGT), pancreatic islet morphology, hepatic steatosis (HS), and blood lipids.

Results:

Both compounds acutely reduced FI in mice without inducing CTA. Chronic GHSR-IA1 increased metabolic rate (MR) in chow-fed mice, suppressed FI, and improved OGT in ZDF rats. Moreover, the progression of islet hyperplasia to fibrosis in ZDF rats slowed down. GHSR-IA2 reduced FI and BW in DIO mice and reduced fasting and stimulated glucose levels compared to pair-fed and vehicle-treated mice. GHSR-IA2-treated DIO mice showed decreased blood lipids. GHSR-IA1 treatment markedly decreased HS in DIO mice.

Conclusions:

Our study demonstrates therapeutic actions of novel ghrelin receptor inverse agonists suggesting a possible potential to treat obesity-related metabolic disorders including diabetes mellitus.

Introduction

Ghrelin controls energy homeostasis and acts via the growth hormone secretagogue receptor (GHS-R) [1,2]. Ghrelin is released from the stomach during fasting and before meals, while feeding or glucose intake reduce its circulating levels [3,4]. The GHS-R is expressed in neurons of the hypothalamic arcuate nucleus that contain the orexigenic neuropeptide Y (NPY) [5]. Ghrelin stimulates food intake in rodents and humans and promotes adiposity [3,6]. Ghrelin inhibits insulin release and leads to insulin

resistance [7-9]. Accordingly, ghrelin-deficient mice show increased insulin secretion and are protected against high-fat diet induced glucose intolerance [7]. Moreover, insulin sensitivity is improved in high-fat diet fed GHS-R knockout mice [10]. In the liver, ghrelin counteracts insulin signaling by promoting the expression of the gluconeogenic enzyme phosphoenolpyruvat-carboxykinase [11]. Further, ghrelin increases lipogenic enzyme activity and inhibits lipid oxidation in white fat [12,13].

The ghrelin signaling pathway is a potential pharmacological target for the treatment of obesity-related metabolic disorders. Most prior approaches were based on a blockade of ghrelin signaling by inhibition of ghrelin acylation or secretion, by inactivation of ghrelin via vaccination or ghrelin binding, or by blockade of the GHS-R by receptor antagonists [14]. However, these therapeutic strategies had limited effects because ghrelin levels are decreased rather than increased in most obese compared to lean subjects [15].

The GHS-R exhibits strong constitutive activity of about 50% of maximum signaling activity [16]. Hence, GHS-R signaling is in part independent of ghrelin action and can therefore only be partially inhibited by pharmacological approaches blocking ghrelin binding to the GHS-R. However, pharmacological inhibition of the constitutive activity of the GHS-R by ghrelin receptor inverse agonists (GHSR-IA) allows suppression of GHS-R signaling below the level achieved by inactivation or antagonism of ghrelin. The first identified GHSR-IA compound was an artificial substance P analog [16]. In our current studies, we explored the effects of two novel ghrelin receptor inverse agonists (GHSR-IA1 and GHSR-IA2) on energy homeostasis, and glucose and lipid metabolism using rodent models for obesity-related metabolic disorders. Both compounds belong to the same class of artificial small molecule compounds (see scaffold in supplementary figure 1). Among a variety of other molecules that were developed, these two compounds exhibited the most promising characteristics with respect to pharmacokinetic parameters and biological activity. GHSR-IA2 is the more advanced compound exhibiting better characteristics and predicted effectiveness. For these reasons, we included both test compounds in our studies. Since the two drugs are closely related in

terms of structure, pharmacology and biological activity, we did not conduct all experiments with both substances to avoid experimental redundancy. In experiments 1-2 (functional receptor studies) both compounds were tested for the demonstration of inverse agonist properties. Experiments 3-5 provide additional in vitro and in vivo proof-of-concept studies supporting inverse agonist action. Furthermore, we aimed to confirm an inhibition of food intake that is not mediated by aversive mechanisms (experiments 6-10). Possible effects on energy expenditure were tested by indirect calorimetry (experiment 11). Finally, we used two models of obesity-related metabolic disease (ZDF rats and DIO mice) to test beneficial effects of repeated GHSR-IA treatment on food intake, body weight and composition, oral glucose tolerance, liver steatosis, pancreatic islet morphology and blood lipids (experiments 12-14).

Materials and methods

Please see supplementary material for more detailed experimental procedures. Experiments were approved by the Veterinary Office of the Canton Zurich, Switzerland (application ZH 63/14).

Experiments 1 and 2: evaluation of ghrelin receptor inverse agonist (EC_{50}) and antagonist (IC_{50}) potency with IP-1 (inositol phosphate) and FLIPR (Fluorescent Imaging Plate Reader) calcium assay

Inverse agonist activity and antagonistic potency of the test compounds were measured in vitro using HEK293 cells expressing the human ghrelin receptor (HEK293/GHSR1a) [17].

Experiment 3: electrophysiological studies

Extracellular single-unit recordings from the medial arcuate nucleus (ArcM) of rats were conducted [18]. Ghrelin responsiveness was tested by superfusing artificial CSF containing ghrelin (Bachem, Bubendorf, Switzerland) at a concentration of 10^{-8} M. Due to a comparatively short stimulation duration of around 6 min and in order to achieve effective stimulation, the inverse agonists were superfused at a concentration of 10^{-6} M, which is above the concentration causing a maximum inhibition in the IP-1 study.

Experiment 4: effect of GHSR-IA1 on fasting-induced c-Fos expression

Mice were randomly assigned to one of four groups: fasted-vehicle, ad libitum-vehicle, fasted-GHSR-IA1 and ad libitum-GHSR-IA1. On the day of the experiment, two groups were fasted for 12 h during the light phase while the other groups were fed ad libitum. A 12 h light phase fasting period has been shown to induce c-Fos expression in the arcuate nucleus [19]. At dark onset, mice received GHSR-IA1 (60 mg/kg) or vehicle (0.5% sodium carboxymethyl cellulose, Sigma-Aldrich, St. Louis,

MO, USA) by gavage. Animals were perfused 2 h later and the brains were processed for c-Fos immunohistochemistry.

Experiment 5: effect of GHSR-IA1 on ghrelin-induced c-Fos expression

Mice were randomly assigned to four groups: saline-vehicle, ghrelin-vehicle, saline-GHSR-IA1 and ghrelin-GHSR-IA1 and injected with ghrelin (2 mg/kg *s.c.*) or saline at dark onset. Thirty minutes later, food intake was measured and mice received a second injection with GHSR-IA1 (30 mg/kg *s.c.*) or vehicle (DMAC 10%/ Solutol 6%/ PBS 84%). Food intake was measured again 30 minutes later. Animals were perfused 2 h after dark onset. Brains were processed for c-Fos immunohistochemistry.

Experiments 6-8: feeding trials

The effect of a single treatment with GHSR-IA1 (30 mg/kg *s.c.* and oral administration of 60 mg/kg, experiments 6 and 7) and GHSR-IA2 (30 mg/kg *s.c.* administration, experiment 8) on food intake was investigated in mice.

Experiments 9 and 10: conditioned taste aversion tests

Mice were water-deprived for 12 h before access to a 0.15% saccharin solution for 2 h. The mice were then injected with vehicle (DMAC 10%/ Solutol 6%/ PBS 84%, 10 ml *s.c.*) as negative control, LiCl (0.15M, 10 ml/kg i.p.) as positive control, GHSR-IA1 or GHSR-IA2 (30 mg/kg *s.c.*, experiments 9 and 10, respectively). Saccharin was removed and the mice had 22 h access to water. On the following day, mice were again water-deprived before access to water and saccharin. Fluid intake was measured after 4 h [20].

Experiment 11: Indirect calorimetry

The effect of GHSR-IA1 on metabolic rate in mice was investigated by indirect calorimetry in an open circuit indirect calorimetry system (TSE Systems, Bad Homburg, Germany) as described previously [21]. Mice were treated daily with GHSR-IA1 (30 mg/kg *s.c.*) or vehicle. Vehicle-treated mice were pair-fed, i.e. they received the same amount of food that GHSR-IA1-treated animals had eaten the previous day. Injections were always given just before dark onset for 6 days.

Experiments 12-14: chronic treatment studies with ZDF rats and DIO mice

ZDF rats were obtained at 5 weeks of age (experiment 12). After adaptation, a baseline oral glucose tolerance test (OGTT) and blood sampling from the sublingual vein was performed. At 8 weeks of age, daily treatment with GHSR-IA1 (20 mg/kg *i.p.*) or vehicle (1 mmol methanesulfonic acid, 1% w/v hydroxyethylcellulose, 0.25% v/v Tween 80, 0.05% v/v antifoam in distilled water) was started.

Two experiments with DIO mice were conducted that were obtained at 17-22 weeks of age. A baseline OGTT was performed after two weeks of adaptation. Three days later, daily treatment with GHSR-IA1 (experiment 13) and GHSR-IA2 (30 mg/kg *s.c.*, experiment 14) or vehicle (1 mmol methanesulfonic acid, 1% w/v hydroxyethylcellulose, 0.25% v/v Tween 80, 0.05% v/v antifoam in distilled water), respectively, was started. Subcutaneous injections were done in mice because they were in better tolerated than i.p. injections. To compensate for possible differences in absorption a higher dose was used for s.c. administration. The vehicle-treated groups were further divided into ad libitum and pair fed mice. Pair-fed mice received the same amount of food that GHSR-IA-treated animals had consumed on the previous day.

Injections were performed 1 h before dark onset for 10 days. OGTTs were performed after 5 and 10 days of treatment. Animals were sacrificed by exsanguination under isoflurane anesthesia one day after the final OGTT. Blood was collected from the right ventricle and pancreas and liver samples were obtained.

Statistics

Data are expressed as means ± SE and analyzed with Student's t-test (paired or unpaired), one-way ANOVA, or Kruskal-Wallis test as appropriate. Tukey's post hoc tests (ANOVA) or Dunn's multiple comparisons test were used to determine differences between groups. A p value of < 0.05 was considered significant.

Results

Experiments 1 and 2: Evaluation of ghrelin inverse agonist potency (EC_{50}) and antagonist potency (IC_{50}) with IP-1 assay and FLIPR Calcium Assay

GHSR-IA1 and GHSR-IA2 reduced the constitutive activity (IP-1 assay) of GHSR expressed in HEK293 with an EC_{50} of 1.7 and 0.4 nM, respectively. The antagonist potencies (IC_{50}) of GHSR-IA1 and GHSR-IA2 (FLIPR assay) were 38 nM and 1.2 nM, respectively (see supplementary figures 1, 2 and 4 for additional compound characteristics).

Experiment 3: GHSR-IA reduced activity of ghrelin-excited neurons

All eight tested neurons were excited by ghrelin while GHSR-IA1 (10^{-6}M) caused a long-lasting and slowly reversible decrease in discharge rate (figure 1A-C). Ghrelin responsiveness was restored after washout of the GHSR-IA1 indicating the absence of receptor desensitization. The inverse agonist activity of GHSR-IA1 contrasted with the effect of a GHS-R antagonist (GHSR-A) characterized in parallel studies because the antagonist blocked ghrelin-induced excitations but did not alter neuronal activity by itself (supplementary figure 3).

Experiments 4 and 5: GHSR-IA reduced fasting- and ghrelin induced c-Fos expression

As described [22-24], 12 h food deprivation during the light phase led to a marked increase in c-Fos positive Arc neurons. Oral administration of the GHSR-IA1 (60 mg/kg) significantly reduced the c-Fos response of fasted mice, but did not induce c-Fos expression in ad libitum fed animals (figure 1D-E). Ghrelin (2 mg/kg *s.c.*) induced a c-Fos response in ad libitum fed mice, which was reversed by GHSR-IA1 (30 mg/kg *s.c.*, figure 1F).

Experiments 5-10: Effects of GHSR-IA treatment on food intake

GHSR-IA1 (30 mg/kg *s.c.*) significantly reduced cumulative food intake for 24h (figure 2A). After oral gavage (60 mg/kg), food intake was only significantly decreased after 1 and 2 h (figure 2B). Subcutaneous injection of GHSR-IA2 reduced food intake by 17% after 24 h and eating was still significantly suppressed after 72 h (figure 2C).

Ghrelin (2 mg/kg *s.c.*) increased 30 min food intake compared to controls by 55% (figure 2D). GHSR-IA1 injected at this time point prevented feeding during the following 30 min almost completely while the ghrelin-injected controls continued to eat, leading to significantly higher 60 min food intake compared to all other groups (figure 2E).

Neither GHSR-IA1 nor GHSR-IA2 induced a CTA. Saccharin consumption did not differ between GHSR-IA1 or GHSR-IA2 and the respective controls, while saccharin intake was significantly lower in LiCl-treated mice. Water intake was not affected any treatment (figure 2F-I).

Experiment 11: Effect of GHSR-IA1 on energy expenditure and respiratory exchange rate (RER)

GHSR-IA1 treatment increased energy expenditure compared to pair-fed mice (figure 3). The effects of GHSR-IA1 treatment on dark and light phase energy expenditure were also evident when data were corrected for body weight, i.e. energy expenditure was only significantly increased during the light phase. There was no effect of repeated GHSR-IA1 treatment on RER in chow-fed mice.

Experiment 12: GHSR-IA1 reduced food intake and improved glucose tolerance in ZDF rats

GHSR-IA1 (20 mg/kg *i.p.*) reduced daily food intake by 10% during the 10-day treatment period compared to vehicle-treated animals (figure 4A). The lower food intake did not lead to a significant decrease in body weight gain (figure 4B). There was a specific decrease in visceral fat mass in GHSR-IA1 treated animals without affecting subcutaneous fat or lean mass (figure 4C-D). Serum free fatty acids and triglyceride levels were reduced by GHSR-IA1, but this effect did not reach statistical significance (figure 4E-F). There was no difference in liver fat content between groups, as determined by Oil red O quantification (data not shown).

After 5 days of GHSR-IA1 treatment, glucose tolerance was significantly improved at individual time points and in the AUC values (figure 4H/J). After 10 days of GHSR-IA1 treatment, this effect was even more pronounced (figure 4I-J). The progression of islet hyperplasia to islet fibrosis was attenuated by GHSR-IA1 reflected by a reduced ratio of fibrotic to hyperplastic islets (figure 4K-M). Terminal insulin values were not significantly different between the groups (see supplements).

Experiments 13 and 14: GHSR-IA treatment reduced food intake, body weight and improved glucose tolerance in DIO mice

Daily treatment with GHSR-IA1 and GHSR-IA2 (30 mg/kg *s.c.*) significantly reduced daily food intake compared to controls (figure 5A-B). Body weight gain was significantly reduced compared to ad libitum fed controls (figure 5C). GHSR-IA2 reduced body weight compared to ad libitum fed controls at the end of the 10-day treatment period (figure 5D). Although pair-fed mice had a higher body weight than the GHSR-IA2-treated mice at that time point, the difference was not statistically significant. However, there were clear differences in body weight change between the three treatment groups (figure 5E). GHSR-IA2-treated mice lost 14.4% of their body weight, while weight loss in pair-fed mice versus ad libitum fed controls was significantly smaller (7.7%) than in the GHSR-IA2

group. Although GHSR-IA2-treated mice had the lowest total and subcutaneous fat mass, the differences did not reach statistical significance (figure 5F-G). Interestingly and similar to the ZDF rats, GHSR-IA2-treated mice had significantly less visceral fat mass than ad libitum fed controls, while lean body mass was almost identical in all groups (figure 5H/I).

Repeated GHSR-IA2 treatment strongly improved OGT after 5 and 10 days of treatment compared to ad libitum fed and pair-fed controls for most time points (figure 5J-L). At day 10, fasting glucose of pair-fed animals was lower than in controls but higher than in GHSR-IA2-treated mice. Improved glucose tolerance was reflected by a lower AUC for OGTT values in the GHSR-IA2 treatment group compared to ad libitum fed controls, although this effect was only significant at day 5. The effect of GHSR-IA1 on oral glucose tolerance was similar to GHSR-IA2, although the treatment effect was less pronounced (supplementary figure 6).

Experiments 13 and 14: IA treatment improved liver steatosis and the blood lipid profile in DIO mice

GHSR-IA1 treatment resulted in a substantial decrease in liver fat content (figure 6A), which was not due to reduced food intake because it did not occur in pair-fed animals. Both GHSR-IA1 and GHSR-IA2 significantly reduced free fatty acid levels (figure 6B/D). Moreover, GHSR-IA2 significantly reduced blood triglycerides (figure 6E). DIO mice that were pair-fed to the GHSR-IA2 group showed similar reduction in free fatty acids and triglycerides. Neither total cholesterol nor HDL values were significantly different among the groups, although there was a tendency of increased HDL in GHSR-IA2-treated mice (supplementary figure 7).

Discussion

GHSR-IA1 inhibited all ghrelin-excited Arc neurons. We confirmed the inhibitory effect of GHSR-IA1 on Arc neurons in immunohistochemical studies. The reversal of fasting-induced c-Fos expression in the Arc is a correlate of neuronal inhibition induced by hormones and metabolic signals such as PYY, leptin, and glucose [25]. GHSR-IA1 not only inhibited fasting-induced but also ghrelin-induced Arc activation. Our *in vitro* and *in vivo* findings are consistent with the general concept defining the mode of action of inverse receptor agonists and competitive receptor antagonists.

Both GHSR-IA1 and GHSR-IA2 inhibited food intake after acute injections. Food intake was still significantly suppressed 72h after GHSR-IA2 treatment. This long-lasting *in vivo* action is most likely a consequence of the long plasma half-life time of 22h. GHSR-IA1 also blunted the feeding response induced by ghrelin, overriding the action of the native receptor ligand. The effects of both test compounds on food intake were not due to aversion because neither GHSR-IA1 nor GHSR-IA2 caused a CTA response.

GHSR-IA1 increased energy expenditure, which is consistent with a decrease of energy expenditure by ghrelin via a suppression sympathetic innervation of brown adipose tissue [26,27] and by inhibiting uncoupling protein 1 (UCP-1) expression [13]. We cannot exclude that differences in physical activity might have influenced energy expenditure. Although cumulative dark phase energy expenditure was not different between the groups, there was a prominent increase in energy expenditure of the GHRS-IA1 group in the second half of the dark phase, which might be activity-related. While RER was not affected by GHSR-IA1, the effect of ghrelin or GHS-R signaling on fat utilization appears to be diet-dependent because a reduction in RER has been observed in GHS-R deficient mice

maintained under high fat diet [28]. We did not test whether GHSR-IA1 treatment reduces RER under high fat diet feeding conditions, which seems plausible based on these findings.

Repeated GHSR-IA1 treatment reduced food intake in ZDF rats. Interestingly, visceral adiposity was decreased by GHSR-IA1 treatment, which is therapeutically more relevant than a reduction of subcutaneous adiposity given the importance of visceral fat depots for metabolic dysregulation [29]. The selective loss of visceral adipose tissue might me due to higher lipolytic or lower anti-lipolytic responsiveness, and reduced lipogenic activity compared to subcutaneous fat, either directly induced by GHSR-IA1 or indirectly via other mechanisms. Blood triglycerides and free fatty acids tended to be reduced in GHSR-IA1 treated ZDF rats. The decreased visceral adiposity and possibly subsequent changes in liver lipid metabolism might have contributed to this tendency. In fact, hypertriglyceridemia has been demonstrated to be the only independent correlate of visceral adiposity among several biometric and metabolic parameters in obese humans [30].

The most striking effect of GHSR-IA1 in ZDF rats was the improvement in OGT. This effect was unrelated to body weight loss, because at least in this model GHSR-IA1 did not significantly affect total body weight, fat or lean mass. The reduction in visceral adiposity and the metabolic consequences discussed above probably do not solely account for the improved glucose tolerance. Acute ghrelin treatment induces peripheral insulin resistance in humans [31]. Therefore, an improvement of insulin sensitivity by GHSR-IA1 possibly contributed to the restoration of glucose tolerance in ZDF rats. In addition, ghrelin signaling exerts a diabetogenic effect in rodents [7,32] and humans [8,9] by inhibiting insulin secretion. It was beyond the scope of our studies to dissociate effects of GHSR-IA1 treatment on insulin sensitivity and insulin secretion, respectively.

The development of pancreatic islet fibrosis is a histopathological hallmark of islet dysfunction. As a compensatory response to hyperglycemia, beta-cells start to proliferate leading to beta-cells hypertrophy and hyperplasia [33,34]. Pancreatic stellate cells are also activated by hyperglycemia and

hyperinsulinemia in an additive manner [35] and secrete extracellular matrix proteins contributing to fibrosis. Interestingly, ZDF rats show a more severe degree and earlier onset of pathological islet characteristics (including fibrosis) than obese non-diabetic Zucker Fatty rats [36]. The amelioration of glucose homeostasis by GHSR-IA1 is a plausible mechanism that might have counteracted the development of islet fibrosis from islet hyperplasia in our studies. The treatment-dependent reduction of fibrotic vs hyperplastic islet without an increase of normal islets indicates that the treatment probably was not able to reverse hyperplasia that pre-existed before the start of treatment. Whether earlier start and longer treatment duration might also prevent the development of islet hyperplasia remains to be demonstrated.

In line with our observations in DIO mice, GHS-R-null mice kept on high-fat diet show reduced food intake supporting the crucial role of constitutive GHS-R activity in the control of feeding behavior [28]. GHSR-IA2-treated DIO mice showed absolute loss of body weight and visceral fat mass. Pair-fed DIO mice tended to show somewhat smaller reductions in body weight and changes in body composition, but the only significant difference compared to GHSR-IA2-treated mice was a smaller change in body weight normalized to pre-treatment values. Therefore, reduction of food intake does not fully explain the treatment-induced change in body weight. Increased energy expenditure protects ghrelin-null mice against high-fat diet induced body weight gain [37]. While GHSR-IA1 increased energy expenditure in chow-fed lean mice, we did not measure possible treatment effects on energy expenditure of GHSR-IA2 in DIO mice. Based on the increased energy expenditure of chow-fed mice under repeated GHRS-IA1 treatment, an increase in metabolic rate might also have contributed to body weight loss in DIO mice.

GHSR-IA2 markedly improved OGTT values, expressed as absolute glucose levels or AUC in DIO mice compared to pair-fed animals. This improvement of the OGTT response is therefore to a great extent independent of food intake. Furthermore, this effect cannot be attributed to differences in

absolute body weight. This became particularly evident after 5 days of treatment, when body weights were similar while OGTT values were much lower in GHSR-IA2-treated vs. pair-fed mice. The mechanisms contributing to treatment-induced improvements of OGT (e.g. increased insulin secretion and/or sensitivity) remain to be investigated. In contrast to our findings in ZDF rats, a pronounced reduction in basal fasting glucose levels occurred during GHSR-IA2 treatment. The lowering of fasting glucose values might be indicative of decreased glucose output from the liver via decreased hepatic gluconeogenesis [38]. Interestingly, food-deprived ghrelin-deficient mice exhibit hypoglycemia which can be reversed by infusion of gluconeogenic precursor substrates [39] suggesting ghrelin-dependent increases in gluconeogenic glucose output from the liver. Hence, a reduction of hepatic glucose output might explain the lowering of fasting-glucose by GHSR-IA2 treatment, which is another beneficial therapeutic mechanism.

GSHR-IA1 treatment strongly reduced liver steatosis in DIO mice. The possible therapeutic value of this treatment effect is substantial because hepatic steatosis is part of a spectrum of pathological changes that are categorized as nonalcoholic fatty liver disease (NAFLD) [40]. Obesity and T2DM are major risk factors for NAFLD-related liver transplantation [41]. A shift of lipids from adipose tissue into ectopic sites, particularly the liver, promotes the development of NAFLD. Insulin resistance plays a crucial role in this process [42]. Chronic ghrelin treatment induces liver steatosis in rats and mice maintained on standard chow and high-fat diet [12], while GHS-R deficient mice are protected against high-fat diet induced liver steatosis. The effect of GHS-R signaling on hepatic steatosis appears to depend on an mTOR-PPAR-γ signaling pathway promoting lipogenesis in hepatocytes [43]. Hence, in addition to improved glucose homeostasis and insulin sensitivity, the protective effect of GHSR-IA1 against liver steatosis could involve a direct inhibition of hepatic lipogenesis.

Finally, the beneficial effects of GHSR-IA treatment on body weight, visceral adiposity and glucose homeostasis were paralleled by improved blood lipid profiles. GHSR-IA2 was more effective because it also significantly reduced the blood triglyceride level. In the GHSR-IA2 studies blood

lipid profiles of pair-fed mice were similar to the treatment group indicating an indirect action probably via the reduction of food intake. Although the effect of GHSR-IA2 treatment on HDL levels did not reach statistical significance in our studies, HDL values might be positively influenced under longer treatment durations (see supplementary figure 7).

In summary, we confirmed inverse ghrelin receptor agonist responses on the functional single cell and *in vivo* level. While both tested compounds showed beneficial effects on energy balance and glucose homeostasis, GHSR-IA2 appeared to be more effective, which is in line with pharmacological profiles. We demonstrated in two rodent models that GHSR-IA are promising therapeutic compounds for the treatment of obesity-related metabolic disorder such as T2DM and comorbidities contributing to the metabolic syndrome.

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Author contributions

T. Riediger: principle investigator, design, preparation of manuscript; Kathrin Abegg, Lara Bernasconi, Melanie Hutter, Lynda Whiting: experiments, analysis, preparation of manuscript; Claudio Pietra, Claudio Giuliano, Thomas A. Lutz: proofreading and discussion of results.

Conflict of interests

Claudio Pietra and Claudio Giuliano are employees of the company Helsinn SA, Lugano, Switzerland.

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Figure legends

Figure 1.

Effect of the inverse ghrelin receptor antagonist GHSR-IA1 on neuronal activity in the arcuate nucleus**.** A) Representative single cell recording of a neuron in the medial arcuate nucleus, which is excited by superfusion (black bars) of ghrelin and inhibited by GHSR-IA1. Ghrelin responsiveness persisted after washout of GHSR-IA1. B-C) Comparison between the effect parameters of the excitatory action of ghrelin and the inhibitory effect of GHSR-IA1. Representative photomicrographs (D) and quantification of cells numbers illustrating the inhibitory effect of GHSR-IA1 on fasting-induced (E) and ghrelin-induced (F) c-Fos expression in the arcuate nucleus. Data analyzed with Student's *t*-test (B-C) or with one-way ANOVA followed by Tukey's post-hoc test (E-F). Means with different letters or symbols are significantly different from each other; $* p < 0.05$, $** p < 0.01$).

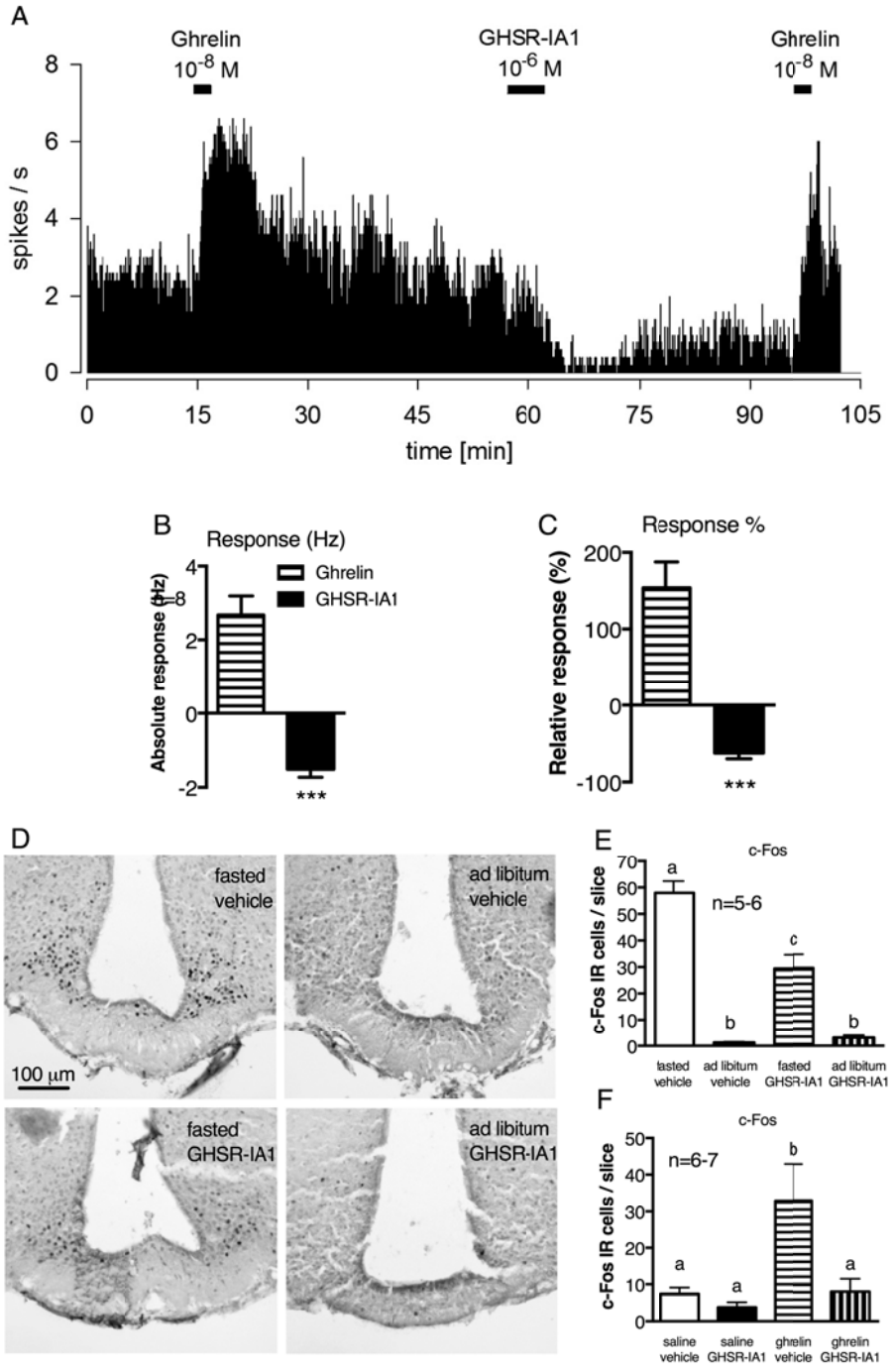

Figure 2.

Effect of the inverse ghrelin receptor antagonists GHSR-IA1 and GHSR-IA2 on food intake**.** GHSR-IA1 inhibited 24-hour food intake after subcutaneous injection. B) GHSR-IA1 significantly reduced feeding for 2 hours after oral administration. C) GHSR-IA2 suppressed food intake for 72h after subcutaneous injection. D-E) GHSR-IA2 treatment counteracted ghrelin-induced increase in food intake. Pre-treatment with ghrelin increased feeding during 30 min (D). GHSR-IA2 treatment 30 min after ghrelin treatment prevented a further increase in food intake measured 60 min after ghrelin treatment (E). While LiCl (positive control) induced a conditioned taste aversion (CTA), neither GHSR-IA1 (F) nor GHSR-IA2 (H) triggered a CTA response as measured by saccharine intake 2 days after a conditioning trial. G/H) Water intake during the test trial was not different between the treatment groups. Data analyzed with Student's *t*-test (A-D) or with one-way ANOVA followed by Tukey's post-hoc test (E-I). Means with different letters or symbols are significantly different from each other; $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$).

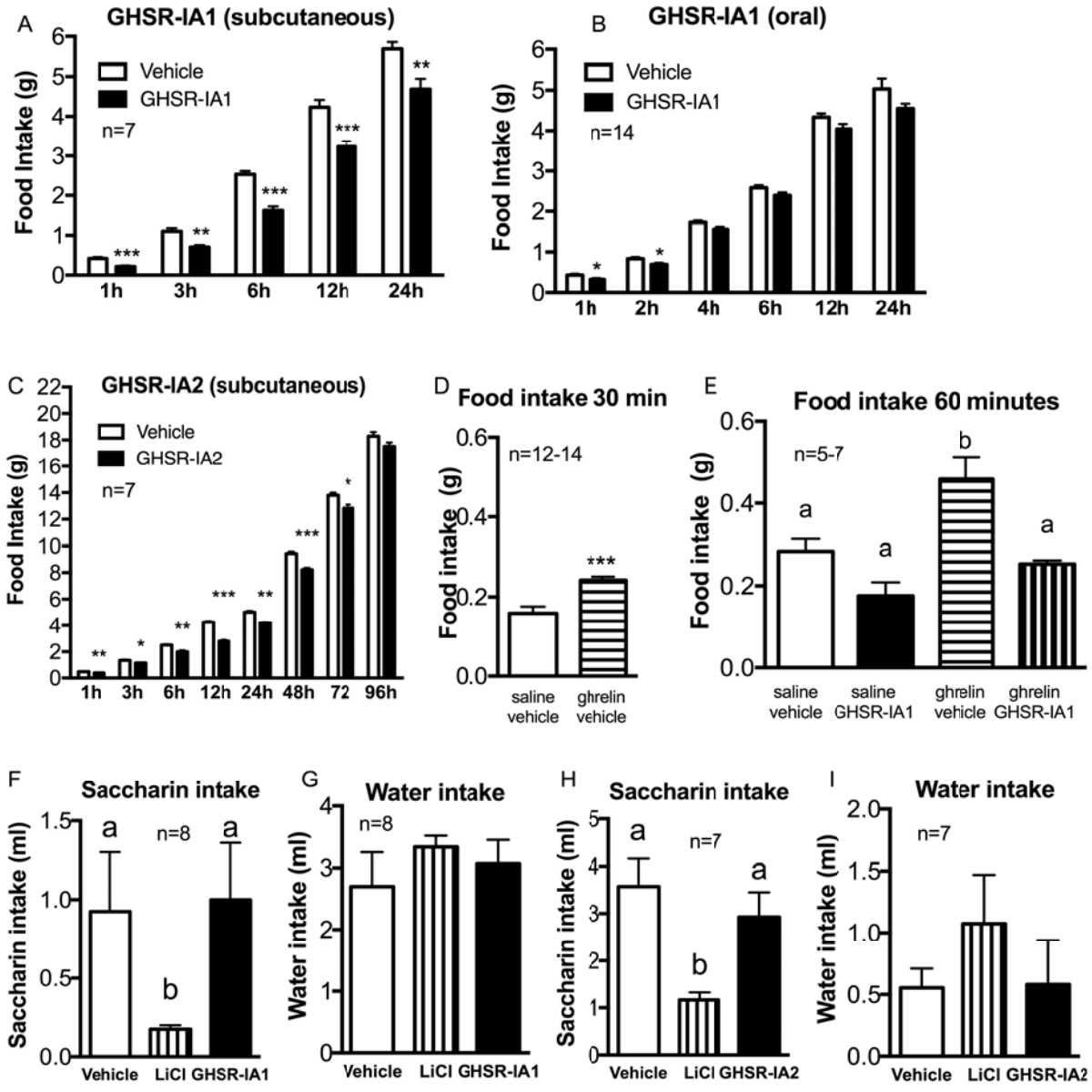

Figure 3.

Daily treatment with GHSR-IA1 increased total cumulative energy expenditure (A) in ad libitum chow fed mice compared to pair-fed controls. This effect was mainly due to an increase of light phase but not dark phase energy expenditure. B) Daily time course of metabolice rate averaged across all treatment days. The average body weights on the final day were 23.3 ± 0.2 g (pair-fed controls) vs. 24.9 ± 0.2 g (GHSR-IA1). Data analyzed with Student's *t*-test (* $p < 0.05$, ** $p <$

0.01; letters: metabolic rate of GHSR-IA1 group was significantly higher (a) or lower (b) than controls).

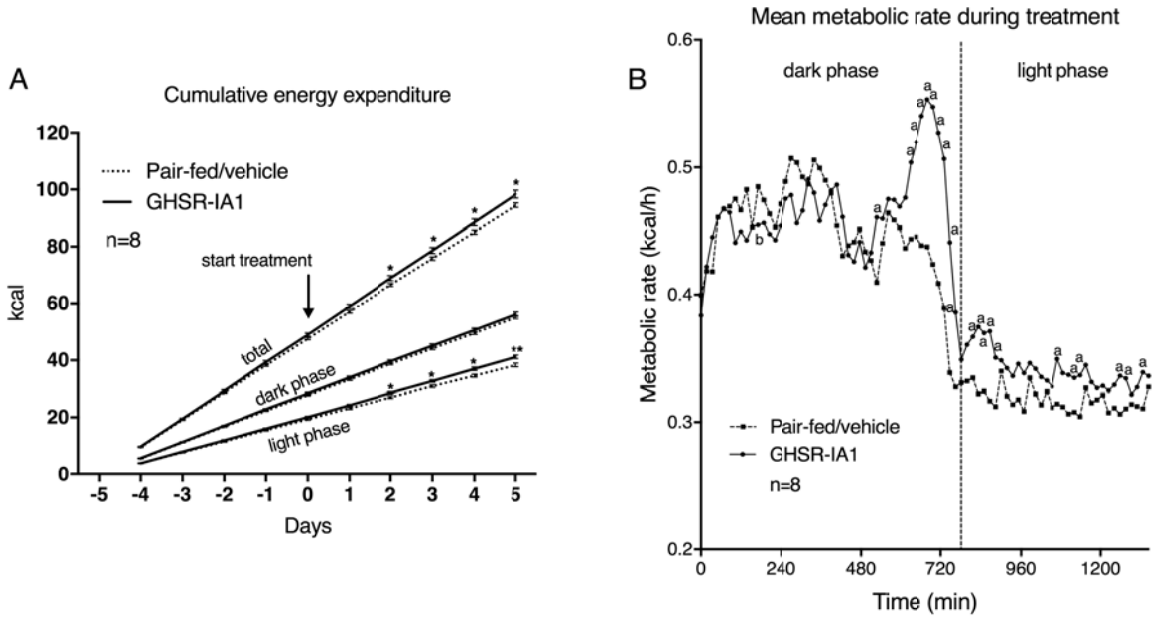

Figure 4.

A-F) Effect of chronic treatment with GHSR-IA1 on food intake, body weight, body composition and blood lipids in ZDF rats maintained on high fat diet. Both experimental groups had similar OGTT values before the start of treatment (G). GHSR-IA1 treated ZDF rats showed improved oral glucose tolerance at treatment days 5 and 10 (H-I). J) Area under the curve values for OGTT responses shown in graphs G-I. K-L) GHSR-IA1 treatment inhibited the progression of islet hyperplasia to islet fibrosis. M) Immunostainings for insulin showing representative examples of different islet morphologies. (A-F, L) Data analyzed using the Student's *t*-test, n=7-8 for all data, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. J) Data analyzed with one-way ANOVA followed by Tukey's post-hoc test *** $p < 0.001$ vs control within OGGT, + $p < 0.05$ vs control of OGGT1, ++ $p < 0.01$ vs control of OGGT1).

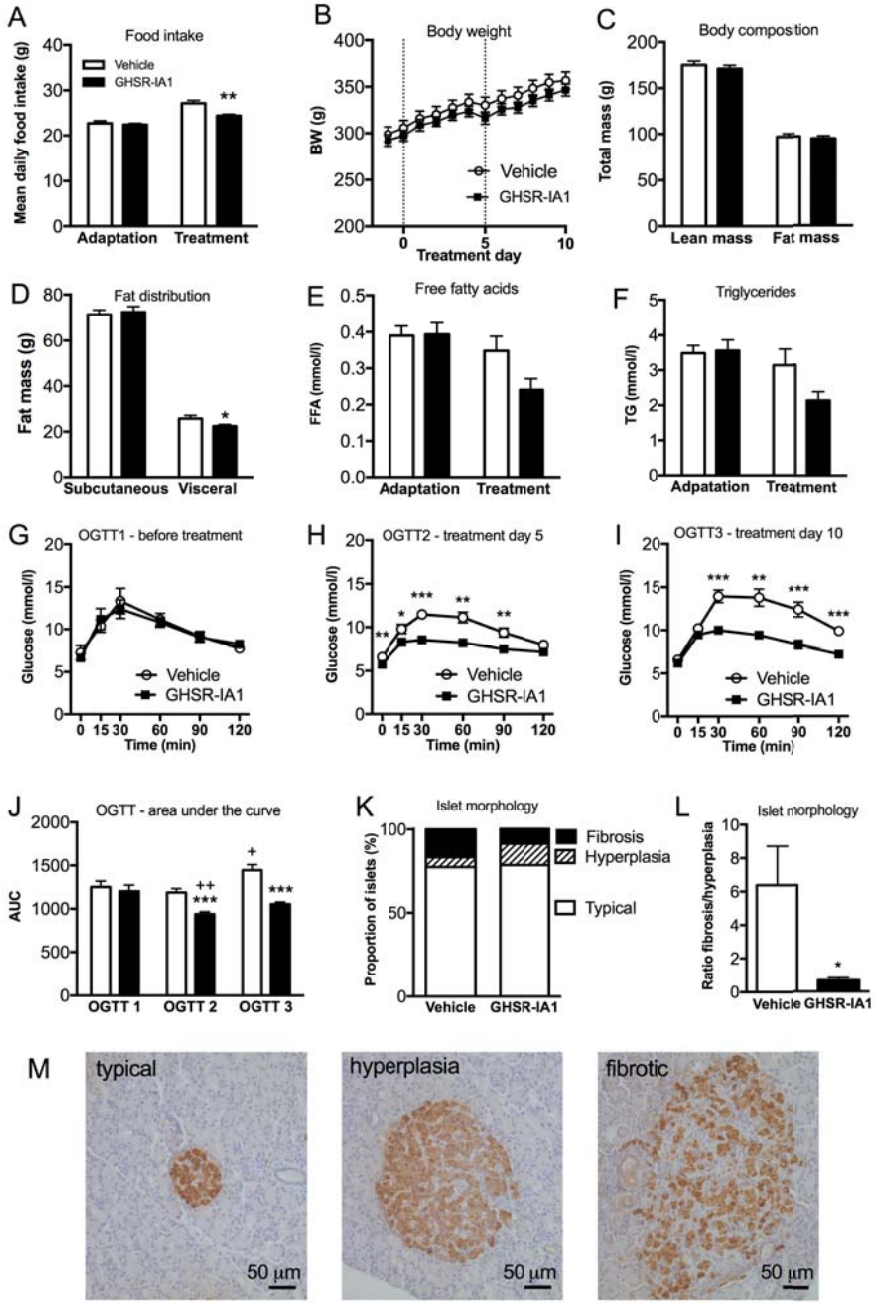

Figure 5.

A-B) Daily food intake of DIO mice maintained on high fat diet during 10-day treatment with GHSR-IA1, GHSR-IA2 or vehicle. C) Body weight change of DIO mice treated with vehicle, GHSR-IA1 and of animals pair-fed to the GHSR-IA1 group. D-E) Body weight and body weight change of DIO mice treated with vehicle, GHSR-IA2 and of animal pair-fed to the GHSR-IA2 group. F-H) Body composition of GHSR-IA2 treated DIO mice and ad libitum and pair-fed controls after 10-

day treatment with GHSR-IA2. J-O) Effect of GHSR-IA2 treatment and pair-feeding on oral glucose tolerance in DIO mice. M-O) Area under the curve values for OGTT responses shown in graphs J-L. A-B) Data analyzed using the Student's *t*-test. C-O) Data analyzed with one-way ANOVA followed by Tukey's post-hoc test or by Kruskal-Wallis test followed by Dunn's multiple comparisons test, respectively. N=9-10 for all data, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; GHSR-IA1 or GHSR-IA2 vs vehicle: a < 0.05, aa $p < 0.01$, aaa $p < 0.001$; accordingly pair-fed/vehicle vs vehicle: b; GHSR-IA1 or GHSR-IA2 vs pair-fed/vehicle: c.

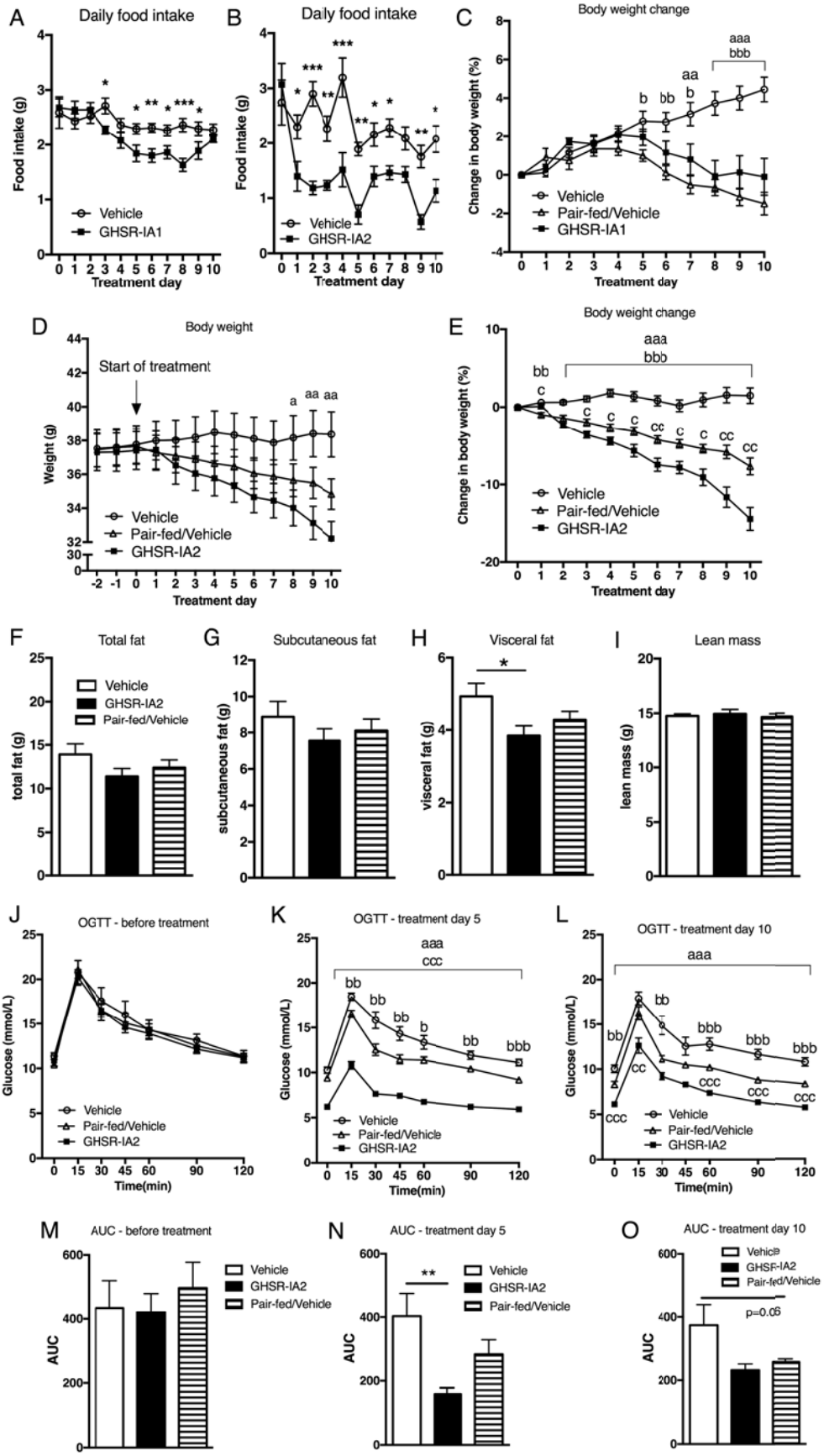

Figure 6.

A) Representative photomicrographs showing Oil red O staining of liver slices from DIO mice treated with vehicle, GHSR-IA1 and of animals pair-fed with the GHSR-IA1 group. Liver fat content as reflected by the Oil red O positive area was significantly lower in GHSR-IA1 treated mice compared to both ad libitum fed and pair-fed control groups**.** B-E) Blood lipids of DIO mice treated with vehicle, GHSR-IA1 or GHSR-IA2, and of animals pair-fed with the pertinent GHSR-IA group. Blood samples were taken at the end of the 10-day treatment period. Data analyzed with one-way ANOVA followed by Tukey's post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).